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Distribution and Clinical Significance of Nuclear Factor Erythroid 2-Related Factor 2 Gene Polymorphism in Chronic Hepatitis B: A Cross-Sectional Study

Distribusi dan Signifikansi Klinis Polimorfisme Gen *Nuclear Factor Erythroid 2-Related Factor 2* pada Hepatitis B Kronis: *A Cross-Sectional Study*

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ABSTRACT

Chronic hepatitis B (CHB) infection is associated with serious complications, including liver cirrhosis and hepatocellular carcinoma (HCC). Nuclear factor erythroid 2-related factor 2 (*NRF2*) is a transcription factor that regulates the expression of antioxidant genes, helping protect cells and tissues from oxidative stress, a process involved in the pathogenesis of chronic hepatitis B (CHB). This study aimed to determine the distribution of *NRF2* polymorphisms in CHB patients and their association with complications. The study included 68 CHB patients, with 33 having no complications and 35 with complications (Cirrhosis and HCC). Genotyping of the *NRF2* polymorphisms, rs35652124 (A→G) and rs6721961 (C→A), was performed using confronting two-pair primers and polymerase chain reaction (PCR-CTPP). The serum levels of bilirubin, albumin, and alanine aminotransferase (ALT) were measured using commercial kits. The mean age of subjects was 45.34±1.32 years old on average. There was no significant difference in mean bilirubin and ALT levels between patients with and without CHB complications. However, patients without complications had significantly higher albumin levels than those with complications (4.0±0.8 vs. 3.37±0.7 g/dL; $p<0.05$). The most common genotypes for NRF2 rs35652124 were AG (51.85%), AA (40.74%), and GG (7.41%), while for NRF2 rs6721961, the were CA (51.47%), CC (45.59%), and AA (2.94%). The distribution of NRF2 genotypes did not differ significantly between CHB patients with and without complications ($p>0.05$). This study suggests that NRF2 gene polymorphisms may not contribute to the development of Cirrhosis and HCC in CHB. Further research with a larger sample size is needed to confirm these findings.

Keywords: Chronic hepatitis B, Cirrhosis, Hepatocellular carcinoma, *NRF2*, polymorphism

ABSTRAK

Infeksi hepatitis B kronis (HBK) berhubungan dengan komplikasi fatal seperti sirosis hati dan karsinoma hepatoseluler (KH). *Nuclear factor erythroid 2-related factor 2* (*NRF2)* adalah faktor transkripsi yang mengatur ekspresi gen antioksidan untuk melindungi sel dan jaringan dari stres oksidatif, yang berperan dalam patogenesis HBK. Penelitian ini bertujuan untuk menentukan distribusi polimorfisme *NRF2* di antara pasien HBK dan hubungannya dengan komplikasi. Studi ini melibatkan 68 pasien HBK, dengan 33 pasien tanpa komplikasi dan 35 pasien dengan komplikasi (sirosis dan KH). Genotipe polimorfisme *NRF2*, rs35652124 (A→G) dan rs6721961 (C→A), dianalisis menggunakan *confronting two-pair primers and polymerase chain reaction* (PCR-CTPP). Kadar bilirubin serum, albumin, dan alanin aminotransferase (ALT) diukur menggunakan kit komersial. Rata-rata usia subjek adalah 45,34±1,32 tahun. Tidak ada perbedaan signifikan pada kadar rata-rata bilirubin dan ALT antara pasien dengan dan tanpa komplikasi HBK. Namun, pasien tanpa komplikasi memiliki kadar albumin yang secara signifikan lebih tinggi dibandingkan dengan pasien dengan komplikasi (4,0±0,8 vs. 3,37±0,7 g/dL; $p<0,05$). Genotipe yang paling umum untuk *NRF2* rs35652124 adalah AG (51,85%), AA (40,74%), dan GG (7,41%), sedangkan untuk *NRF2* rs6721961 adalah CA (51,47%), CC (45,59%), dan AA (2,94%). Distribusi genotipe *NRF2* tidak berbeda secara signifikan antara pasien HBK dengan tanpa komplikasi ($p>0,05$). Studi ini menunjukkan bahwa polimorfisme gen *NRF2* mungkin tidak berkontribusi pada perkembangan sirosis dan HCC pada hepatitis B kronis. Penelitian lebih lanjut dengan ukuran sampel lebih besar diperlukan untuk mengonfirmasi temuan ini.

Kata Kunci: Hepatitis B kronis, Sirosis, Karsinoma hepatoseluler, *NRF2*, Polimorfisme

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INTRODUCTION

Chronic hepatitis B (CHB) is a significant global health concern affecting millions of individuals worldwide. Without appropriate treatment, 15–40% of CHB patients may develop Cirrhosis, which can progress to liver failure and hepatocellular carcinoma (HCC) (Fung et al., 2014). Although the mortality rate of CHB has been decreasing in recent decades, CHB infection is responsible for approximately 900,000 deaths annually worldwide (McMahon, 2018). The prevalence of hepatitis B virus (HBV) infection varies across regions, with some parts of Asia and Africa having carrier rates as high as 5-10%, while North America and Western Europe have a prevalence below 1% (Gnyawali et al., 2022). In Indonesia, the endemicity of HBV is classified as moderate to high, ranging from 2.5% to 10%, based on the seroprevalence of hepatitis B surface antigen (HBsAg) (Yano, 2015).

Oxidative stress plays a significant role in the progression of chronic hepatitis B (CHB) (Allameh et al., 2023). Studies have shown that malondialdehyde (MDA) and total oxidative stress (TOS) levels are significantly higher in patients with CHB than in healthy controls (Ciragil et al., 2010; Duygu et al., 2012). The severity of liver disease in CHB is associated with increased oxidative stress, with cirrhosis patients showing lower total antioxidant responses and higher total peroxide levels than inactive carriers (Bolukbas et al., 2005). Oxidative stress is associated with liver injury, with higher levels observed in patients with elevated ALT (Duygu et al., 2012). The imbalance between oxidative stress and antioxidant capacity in CHB patients suggests that monitoring oxidative stress markers may be valuable for evaluating and managing the disease. Oxidative stress induces nuclear factor erythroid 2-related factor 2 (NRF2) activation, facilitating detoxification and eliminating potentially hazardous oxidants. NRF2 expression has been observed in all human tissues; however, it is powerful in the liver and other detoxifying organs. In liver cells, NRF2 is a key regulator of several pathways linked to cellular defence mechanisms against oxidative stress (Tang, 2014).

Understanding the genetic factors that contribute to the progression of chronic hepatitis B to Cirrhosis and hepatocellular carcinoma is crucial for developing effective prevention and treatment strategies (Akcay et al., 2018). Previous studies have identified several genetic factors that may contribute to the development of complications in patients with chronic hepatitis B, including polymorphisms in the NRF2 gene. There have been reports of many single nucleotide polymorphisms (SNPs) in NRF2, such as rs35652124 (A→G) and rs6721961 (C→A) (Ishikawa, 2014). SNPs in NRF2 are thought to function as prognostic indicators for several illnesses, including cancer (Cho et al., 2015). Despite this, the distribution of NRF2 polymorphisms in chronic hepatitis B patients and their relationship with complications, such as Cirrhosis and hepatocellular carcinoma, is not well understood. Thus, this study aimed to explore the distribution of NRF2 polymorphisms in patients with chronic hepatitis B and examine their association with complications such as Cirrhosis and hepatocellular carcinoma.

MATERIALS AND METHODS

Study subjects

This cross-sectional study was conducted at Arifin Achmad Hospital in Riau Province between March and September 2019. Study participants were recruited from the Department of Internal Medicine and categorized into those with complications, such as Cirrhosis, HCC, and CHB without complications. Chronic hepatitis B was diagnosed based on a positive HBsAg result for a minimum of six months. Cirrhosis was diagnosed using imaging (transient elastography and/or ultrasonography) and clinical symptoms, including esophageal varices and ascites. The inclusion criteria consisted of HBV patients aged 18 years or older.

In contrast, the exclusion criteria included co-infection with other viruses, such as hepatitis C and hepatitis D. Serum levels of alanine aminotransferase (ALT), albumin, and bilirubin were measured using commercial kits. Genotype analysis for NRF2 rs35652124 (A→G) was performed on 27 CHB patients, including nine patients without complications and 18 patients with chronic hepatitis B who had complications such as Cirrhosis and/or HCC. Genotyping of NRF2 rs6721961 (C→A) was performed on 68 CHB patients, comprising 33 CHB patients without complications and 35 patients with Cirrhosis and/or HCC complications.

Genotyping of NRF2 Polymorphism

Ten milliliters of venous blood was obtained from each participant and preserved in EDTA tubes. The blood was then centrifuged at 2000 × g for 5 min, the plasma was stored at -80°C, and the blood cells were stored at -20°C for genomic DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega Inc., Madison,

WI, USA). The NRF2 polymorphism was genotyped using a polymerase chain reaction with two pairs of primers (PCR-CTPP) (Table 1) (Shimoyama et al., 2014). The PCR reaction (25 µL) included 12.5 µL of GoTaq® Green Master Mix (Promega Inc., Madison, WI, USA), 0.5 µL each of primers (0.5 µM concentration), 1 µL of template DNA (100 ng), and nuclease-free water to reach the final volume. Thermal cycling conditions for NRF2 rs35652124 included an initial denaturation at 95ºC for 1 min, followed by 30 cycles of denaturation at 95ºC for 40 s, annealing at 63ºC for 40 s, extension at 72ºC for 40 s, and a final extension at 72ºC for 1 min. Similar PCR conditions were used for NRF2 rs6721961 genotyping, except that the annealing temperature was 58ºC. PCR products were visualized by electrophoresis on a 2% agarose gel stained with GelRed® Nucleic Acid Gel (Biotium) in 1x TAE buffer at 75 volts for 40 min and analyzed using UV GelDocTM (Bio-Rad, USA).

Table 1. Primers Used to Detect NRF2 Polymorphisms (Shimoyama et al., 2014).

Gene/ ID SNP	Primers	Genotype
NRF2 rs35652124 (A→G)	F1: CTTTTATCTCACTTTACCGCCCGAG F2: GCAGTCACCCTGAACGCCCT R1: GACACGTGGGAGTTCAGAGGG R2: GGGGTTCCCGTTTTTCTCCC	AA: 317, 145 bp AG: 317, 212, 145 bp GG: 317, 212 bp
NRF2 rs6721961 (C→A)	F1: CCCTGATTTGGAGGTGCAGAACC F2: GGGGAGATGTGGACAGCG R1: GCGAACACGAGCTGCCGGA R2: CTCCGTTTGCCTTTGACGAC	CC: 282, 113 bp CA: 282, 205, 113 bp AA: 282, 205 bp

Statistical analysis

Numerical variables are presented as mean ± SD (min-max), while categorical data are shown as proportions. The Chi-square test assessed group differences in NRF2 polymorphism genotypes and allele frequencies. This test is appropriate for comparing categorical data across multiple groups, assuming that the expected frequency in each category is sufficient. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using SPSS Version 22 (IBM Corp., Armonk, NY, USA).

RESULT AND DISCUSSION

Clinical characteristics of study subjects

The characteristics of the participants are listed in Table 2. Of 68 participants, the mean age was 45.34±13.23 years, and a minimum-maximum age range of 19-73 years. The study included 38 males (56%) and 30 females (44%). The study subjects comprised 33 CHB patients without complications (49%) and 35 CHB patients with complications (51%). Table 3 shows the liver function test results among CHB patients. The results show no significant differences in the mean ALT and bilirubin between the two groups ($p>0.05$). However, the mean albumin level was significantly higher in the CHB group than in the complications ($p<0.002$). Individuals with CHB have an increased risk of developing Cirrhosis and HCC compared with healthy subjects (Trépo et al., 2014). Several factors influence the risk of developing Cirrhosis and HCC in HBV-infected patients, and genetic variants may contribute to the pathogenesis of these complications (Arfianti et al., 2021). Studying the genetic factors will improve our understanding of how various biological pathways play a role in liver carcinogenesis (Nahon & Zucman-Rossi, 2012). This study is crucial for forecasting and managing the risk of Cirrhosis and HCC in individuals with HBV infection.

Table 2. Characteristics of study subjects

Variable	N=68	%
Age		
Mean±SD (Years)	45,34±13,23	
Min-max (Years)	19-73	
Sex		
Male	38	56
Female	30	44
Diagnosis		
CHB	33	49
CHB with complication	35	51

Table 3. Comparison of liver function tests among patients with CHB

Liver function tests	CHB (n=33)	CHB with Complications (n=35)	*p*
ALT (Means±SD, IU/L)	74,59(189,369)	48,10(47,485)	0.460
Bilirubin (Means±SD, mg/dL)	1,29(3,268)	2,03(2,146)	0.305
Albumin (Means±SD, g/dL)	4,0(0,816)	3,37(0,718)	0.002*

Note: ALT, alanine aminotransferase; CHB, chronic hepatitis B. Chi-square test was used to assess this association

NRF2 Polymorphism Genotypes

Table 4 presents the NRF2 genotype frequencies among the CHB patients. The table shows the frequencies of various genotypes and their association with CHB complications. The frequencies of rs35652124 AA, AG, and GG in patients with CHB were 40.74%, 51.85%, and 7.41%, respectively. The AA and AG genotypes were found more frequently in patients with complications than those without complications (54.55% and 71.43%, respectively), whereas the GG genotype was found only in CHB patients with complications. Moreover, the frequencies of rs6721961 AA, CA, and CC in patients with CHB were 2.94%, 51.47%, and 45.59%, respectively. The frequency of the CA genotype was higher in CHB patients with complications (51.43%) than in those without complications (48.57%). In contrast, the frequency of the CA genotype was higher in CHB patients without complications (54.83%) than in those with complications (45.16%), while the frequency of the AA genotype was similar between these two groups. Despite these differences, the distribution of the rs35652124 and rs6721961 genotypes was not significantly different between the two groups ($p > 0.05$). These findings suggest that the genetic polymorphisms of *NRF2* are not significantly linked to the risk of Cirrhosis and HCC in CHB patients, and to the best of our knowledge, only one study in a Chinese population has examined the role of *NRF2* polymorphisms as risk predictors for HBV-related liver cirrhosis and HCC (Liu et al., 2023). Concerning rs6721961, the study found that patients with the GT genotype had a 2.3-fold higher risk of developing HCC than those with the GG genotype (95% confidence interval [CI] = (1.3–4.1); p=0.005). The researchers also investigated the association between *NRF2* rs6726395 polymorphism and the risk of HCC and Cirrhosis; however, no statistically significant difference was observed among the study groups. This discrepancy highlights the need to explore further genetic factors that influence chronic hepatitis B outcomes.

Table 4. NRF2 genotype frequencies among patients with Chronic Hepatitis B patients

NRF2 polymorphisms	Genotype	CHB	CHB with complications	Total	*p*
rs35652124	AA	5 (45.45%)	6 (54.55%)	11 (40.74%)	p>0.05
	AG	4 (28.57%)	10 (71.43%)	14 (51.85%)	
	GG	0 (0%)	2 (100%)	2 (7.41%)	
rs6721961	AA	1 (50%)	1 (50%)	2 (2.94%)	p>0.05
	CA	17 (48,57%)	18 (51,43%)	35 (51.47%)	
	CC	17 (54,83%)	14 (45,16%)	31 (45.59%)	

Note: CHB, chronic hepatitis B. A Chi-square test was used to assess this association.

The involvement of NRF2 genetic variants has also been explored in other liver diseases, including alcoholic liver disease and drug-induced liver injury. Santos et al. (2019) reported that the rs35652124 polymorphism in the NRF2 promoter region is associated with a risk of developing Cirrhosis in patients with alcoholic liver disease (ALD) (Santos et al., 2019). Moreover, a study discovered that NRF2 polymorphisms influence liver injury due to oxidative stress induced by anti-tuberculosis drugs (Chen et al., 2019). In addition to liver diseases, NRF2 polymorphisms also play a role in the development of several diseases, including cardiovascular diseases, neurodegenerative diseases, and cancer. A study on kidney cancer concluded that the NRF2 polymorphism rs6721961 influences cancer development (Cho et al., 2015; Yamaguchi et al., 2019).

Several studies have demonstrated that NRF2 signalling plays a significant role in hepatocarcinogenesis by driving cell proliferation, initiating angiogenesis and invasion, and inducing drug resistance (Kalantari et al., 2023). Therefore, NRF2 targeting presents a potential approach for HCC treatment. A preclinical study demonstrated that Nrf2 ablation reduces histone acetylation, specifically in tumors, inhibiting tumor progression. However, approximately 14% of HCC patients carry mutations in NRF2 or Kelch-like ECH-associated protein 1 (KEAP1), a cellular inhibitor of NRF2 (Cleary et al., 2013). A recent study demonstrated that a gain-of-function mutation in NRF2 decreases the expression of stimulator of interferon genes (STING), a signalling molecule that regulates the transcription of genes crucial for the innate immune response (Li et al., 2024). This study underscores that NRF2 mutations substantially reduce the efficacy of immunotherapy that activates STING. This finding highlights the importance of considering NRF2 mutation status when utilizing STING agonists for hepatocellular carcinoma (HCC) therapies to enhance their efficacy. These findings emphasize NRF2's potential importance in HCC treatment strategies and the need for additional investigations.

A limitation of this study was its relatively small sample size; therefore, the results should be interpreted with caution. Nevertheless, the study may provide significant data on the role of NRF2 in the development of Cirrhosis and HCC in CHB patients, which remains unclear as existing evidence is limited. Second, our study did not include other NRF2 SNPs; therefore, the inclusion of additional SNPs with an expanded sample size would

provide more comprehensive information regarding the influence of NRF2 Polymorphisms and their impact on the risk of Cirrhosis and HCC in patients with CHB.

CONCLUSION

This study suggests that genetic variations in NRF2 may not significantly impact the development of complications associated with CHB infection, including liver cirrhosis and HCC. Although NRF2 polymorphisms are potential risk predictors, the progression of liver disease is influenced by multiple and complex genetic and environmental factors. Consequently, additional studies with larger study populations are required to confirm the impact of NRF2 polymorphisms on the risk of developing Cirrhosis and HCC in chronic hepatitis B patients.

REFERENCES

Akcay, I.M., Katrinli, S., Ozdil, K., Doganay, G.D., Doganay, L., 2018. Host genetic factors affecting hepatitis B infection outcomes: Insights from genome-wide association studies. *World Journal of Gastroenterology*, 24(30): 3347.

Allameh, A., Niayesh-Mehr, R., Aliarab, A., Sebastiani, G., Pantopoulos, K., 2023. Oxidative stress in liver pathophysiology and disease. *Antioxidants*, 12 (9): 1653.

Arfianti, A., Sabillah, S.A., Andrini, D.F., Kartika, S.D., Julia P.A., 2021. Genotype distribution of vitamin D receptor polymorphisms among Indonesian patients with chronic hepatitis B. *Reports of Biochemistry & Molecular Biology*, 9(4):463-469.

Bolukbas, C., Bolukbas, F.F., Horoz, M., Aslan, M., Celik, H., Erel, O., 2005. Increased oxidative stress associated with the severity of the liver disease in various forms of hepatitis B virus infection, *BMC Infectious Diseases*, (5): 95.

Chen, S., Pan, H., Chen, Y., Lu, L., He, X., Chen, H., Chen, R., et al., 2019. Association between genetic polymorphisms of NRF2, KEAP1, MAFF, MAFK and anti-tuberculosis drug-induced liver injury: a nested case-control study. *Scientific Reports*, (9): 14311.

Cho, H.-Y., Marzec, J., Kleeberger, S.R., 2015. Functional polymorphisms in NRF2: implications for human disease. *Free Radical Biology and Medicine*, (88): 362–372.

Çıragil, P., Kurutaş, E. B., Kökoğlu, Ö. F., Aral, M., 2011. Oxidative stress in patients with chronic hepatitis B and C. *Balkan Medical Journal*, 2011(3): 300-303.

Cleary, S.P., Jeck, W.R., Zhao, X., Chen, K., Selitsky, S.R., Savich, G.L., Tan, T.-X., et al., 2013. Identification of driver genes in hepatocellular carcinoma by exome sequencing. *Hepatology (Baltimore, Md.)*, 58(5):1693–1702.

Duygu, F., Karsen, H., Aksoy, N., Taskin, A., 2012. Relationship of oxidative stress in hepatitis B infection activity with HBV DNA and fibrosis. *Annals of Laboratory Medicine*, 32(2): 113–118.

Fung, J., Lai, C.-L., Yuen, M.-F., 2014. Management of chronic hepatitis B in severe liver disease. *World Journal of Gastroenterology : WJG*, 20(43): 16053.

Gnyawali, B., Pusateri, A., Nickerson, A., Jalil, S., Mumtaz, K., 2022. Epidemiologic and socioeconomic factors impacting hepatitis B virus and related hepatocellular carcinoma, *World Journal of Gastroenterology*, 28(29): 3793.

Ishikawa, T., 2014. Genetic polymorphism in the NRF2 gene as a prognosis marker for cancer chemotherapy. *Frontiers in Genetics*, (5): 383.

Kalantari, L., Ghotbabadi, Z.R., Gholipour, A., Ehymayed, H.M., Najafiyan, B., Amirlou, P., Yasamineh, S., et al., 2023. A state-of-the-art review on the NRF2 in Hepatitis virus-associated liver cancer. *Cell Communication and Signaling*, 21(1): 318.

Li, C., Liang, G., Yan, K., Wang, Y., 2024. NRF2 mutation enhances the immune escape of hepatocellular carcinoma by reducing STING activation. *Biochemical and Biophysical Research Communications*, (698):149536.

Liu, Y., Wu, Q., Zhang, F. and Qin, X., 2023. Antioxidants-related nuclear factor erythroid 2-related factor 2 gene variants associated with HBV-related liver disease. *Cancer Cell International*, 23(1):72.

McMahon, B.J., 2018. Meeting the WHO and US goals to eliminate Hepatitis B infection by 2030: Opportunities and challenges, *clinical liver disease*, 12(1):29.

Nahon, P., Zucman-Rossi, J., 2012. Single nucleotide polymorphisms and risk of hepatocellular carcinoma in Cirrhosis, *Journal of Hepatology*, 57(3): 663–674.

Santos, K.N.D., Florentino, R.M., França, A., Lima Filho, A.C.M., Santos, M.L.D., Missiaggia, D., Fonseca, M. de C., et al., 2019. Polymorphism in the promoter region of NFE2L2 gene is a genetic marker of susceptibility to Cirrhosis associated with alcohol abuse. *International Journal of Molecular Sciences*, 20(14): 3589.

Shimoyama, Y., Mitsuda, Y., Tsuruta, Y., Hamajima, N., Niwa, T., 2014. Polymorphism of *NRF2*, an antioxidative gene, is associated with blood pressure and cardiovascular mortality in hemodialysis patients. *International Journal of Medical Sciences*, 11(7): 726–731.

Tang, W., 2014. Role of NRF2 in chronic liver disease, *World Journal of Gastroenterology*, 20(36): 13079

Trépo, C., Chan, H.L.Y., Lok, A., 2014. Hepatitis B virus infection, *Lancet (London, England)*, 384(9959): 2053–2063.

Yamaguchi, Y., Kamai, T., Higashi, S., Murakami, S., Arai, K., Shirataki, H., Yoshida, K.-I., 2019. NRF2 gene mutation and single nucleotide polymorphism rs6721961 of the NRF2 promoter region in renal cell cancer. *BMC Cancer*, (19):1137.

Yano, Y., 2015. Hepatitis B virus infection in Indonesia. *World Journal of Gastroenterology*, 21(38): 10714.